

### **Instructions for the care and use of the Multisizer 3**

The Multisizer is intended for quantifying either cells or spheres. We are in possession of two apertures: one with a 100- $\mu\text{m}$  diameter opening and the other with a 560- $\mu\text{m}$  diameter opening. The default on the Multisizer is the 560- $\mu\text{m}$  aperture which is used for counting spheres. If you need to use the 100  $\mu\text{m}$  aperture for counting cells you need to change two things: first the diluent should be changed to regular Isoton II and secondly, the aperture will need to be recalibrated for the new diluent and aperture size. Please see section 4.4 in the operator's manual for changing the aperture tube and section 5 for calibration of the new aperture.

All of the following instructions assume the use of the 560- $\mu\text{m}$  aperture.

#### ***Things to consider before starting a run on the Multisizer 3***

##### **Do you have enough diluent?**

Life would be easy if we just had to use Isoton II as the diluent but because we are using the large aperture we use the 6:4 Isoton II:Glycerol diluent. This is to reduce the noise in the diluent due to the high rate of intake flow that would occur with just the Isoton II alone. For the same reason, this will also help to keep the spheres from breaking apart while going through the aperture by slowing them down.

Note that if you run 6 collections for each sample as indicated below you will need about 50 ml of diluent for each sample. I would make sure there is at least 1 liter of diluent for each 12 or so samples you plan to run to be safe.

##### **To make diluent (2L):**

Mix 920 ml of 87% glycerol (on shelf) with 1080 ml of Isoton II (in cabinet), stir in a flask until homogenized (the glycerol will sink).

Filter to remove small particulates that will cause noise in the diluent, I usually use a 0.2  $\mu\text{m}$  bottle-top filter (you can re-use it until the whole 2L is filtered), or we also have circular filters in the Multisizer accessories drawer that will work, though you will have to apply vacuum pressure to use these as well (the solution is too viscous to go through on its own).

Transfer filtered diluent to a vacuum flask if not already in one and apply vacuum pressure to de-gas the diluent (you should see millions of tiny bubbles rise to the surface). This will take 20-60 minutes (or it can go longer).

The diluent can be left in the vacuum flask or transferred to clean bottles by gently pouring to minimize more bubbles. It is best if the diluent is allowed to settle overnight before use to allow any bubbles to settle.

##### **Are you spheres too big?**

This should not be a concern if you are working with primary cells but the tumorspheres can get quite large. The aperture is really only accurate at reading between a range of 2-60% of its size, about 11  $\mu\text{m}$  to 336  $\mu\text{m}$ . Large spheres can also clog the aperture, which would be very bad.

Several potential ways to address this:

1. Verify that you large spheres are okay for the multisizer.

I would take a picture of some of the big ones and then use the “Add measurement” feature in the Spot software (EDIT menu) to check the size of these spheres.

2. Alter the conditions of your experiment or run the tumorspheres earlier so they don't grow so large.

Abnormally large spheres in my experience are more likely to result from initial aggregation so plating at a lower density may help with this. Monitor your spheres daily and count them on a day when the size is manageable (older spheres may just keep getting bigger).

3. Try to filter out the large spheres.

We don't have a suitable basket filter or other type of filter that would do this currently (our largest is 100  $\mu\text{m}$ ) but I know that larger ones do exist and could be found with some research.

### **Are your spheres too concentrated?**

Again, this is likely going to be a problem only for the Tumorspheres. Note that in the panel to the left when you run a sample there will be an indicator of the concentration of the events. The multisizer seems to like the concentration between 0-10%, anything higher and you get a red bar. In Chris' experience, when she had too high of a concentration, the machine would behave strangely, counting many, many thousands of spheres and not showing a decay with dilution of the sample. It worked better when she diluted the samples or started with less concentrated samples. We don't know why this is exactly but if you notice that you are running samples at a high >10% concentration then I would suggest that you dilute your sample or plate cells at a lower density to start with.

### **What is the size cutoff that counts as a 'sphere'? What trend can I expect?**

1. I strongly recommend that you take some pictures of your spheres and do some measurements on them even if you don't think you will have size issue as above. This will give you an idea of where you want to put your cutoff when doing data analysis (this only needs to be done once). Try to get a field with small and large spheres. Determine what 'counts' as a sphere in your assay, we should be consistent between users so those using the same cell line should consult each other. Also it is a good idea to take some of your spheres and take pictures of them in the diluent for comparison. I have found that my primary spheres shrink considerably in the viscous diluent-about 15  $\mu\text{m}$  in diameter on average.

Some examples from my primary spheres:

Single cells are about 5  $\mu\text{m}$  in diameter in media

Enlarged cells and doublets (entosis victims?) are about 15-20  $\mu\text{m}$  in diameter in media

Multicellular 'spheres' are about 35-40+  $\mu\text{m}$  in diameter, with most around 50  $\mu\text{m}$  in media

Multicellular 'spheres' are about 20-25+  $\mu\text{m}$  in diameter in diluent, I usually set my counting cutoff at 25-30  $\mu\text{m}$  during data analysis; the consensus for tumorsphere users is about 40  $\mu\text{m}$  for cutoff.

2. If you have different conditions, I would look carefully at your wells/plates before running the samples to see what the expected trend should be (sample A has more spheres than sample B). I sometimes will do a hand count of one well to estimate this trend for my own piece of mind. The multisizer count will not be exactly the same as that by hand for several reasons (human error, the entire sample is not counted by the multisizer, sample loss during preparation etc.) but the trend should be the same. The multisizer has pleasantly always replicated the trend I see and is usually very consistent between replicates.

**Lastly**, remember that spheres run through the multisizer are gone forever so any additional analysis you need to do on the spheres (staining, dissociation for secondary spheres etc.) will need to be done with spheres you set up in parallel to the ones you use for the multisizer. I like to run my samples in triplicate so plan accordingly.

OK, now for operation of the machine! Note: it can be a bit fussy...it helps if it is properly warmed up and you pipet slowly and methodically.

### **Operation of the Multisizer 3**

#### **1. Exchanging Coulter Cleanz storage solution for diluent; preparing for run**

- a. Turn on the Multisizer with the power switch on the Right side, allow it to warm up for 20 minutes or so. I usually use this time to fill multiple coulter counter buckets with diluent. Fill as many as needed for your samples plus extras with 20 ml, pipetting carefully to avoid bubbles. Cover with caps if it will be a long time before you actually get to your samples to limit dust.
- b. Open the software on the desktop (Multisizer 3), make sure the check box with "Connect to Multisizer 3" is checked, then click OK.
- c. Loosen the connection of the Coulter Cleanz bucket (front) on the left side of the machine, pull the tube out from the machine and leave it disconnected.
- d. Go to SYSTEM→DRAIN SYSTEM, click OK on the text box that reminds you to disconnect the tube.

- e. After the system is drained, unscrew the cap from the bottle of coulter cleanz, hose off the wand briefly with dH2O and transfer the cap to the bottle of diluent that is stored to the left of the machine. Make sure to add more diluent slowly if the level looks low. Tighten the cap and reconnect the tube to the side of the machine, push in firmly and re-screw in snugly.
- f. Exchange the coulter bucket of coulter cleanz on the stage for a bucket containing diluent. Press the button on the bottom of the front of the stage to raise and lower it.
- g. Got to SYSTEM→FILL SYSTEM, the blue coulter cleanz should be exchanged for the clear diluent.
- h. Add more diluent to the bucket on the stage (always raise and lower the stage to add diluent to avoid bumping the aperture or the electrode, pipet against the side of the bucket to reduce bubbles). Repeat step g.
- i. Add more diluent to the bucket, flush the aperture tube to get rid of bubbles in the aperture: SYSTEM→FLUSH APERTURE TUBE
- j. Exchange the bucket on the stage for a fresh bucket of diluent, place used buckets in the sink to avoid confusion.
- k. Check the internal waste tank indicator on the bottom left of the screen, if the blue is far to the right or the red is indicted, empty the internal tank: SYSTEM→EMPTY WASTE TANK. This will take a few minutes. Note the machine will not run if the internal tank is full. If the WASTE TANK FULL error message comes up, check this indicator and also the external tank and empty the appropriate one.
- l. Check the level in the external waste tank, if this is near full, disconnect it and empty it into the sink, then replace it.

## 2. Setting up file storage and naming; diluent quality control I

- a. Designate a place for your files to save. Use the shortcut folder on the desktop to get to the multisizer samples file folder. Make yourself a folder if you don't already have one and make a subfolder within this folder for this experiment.
- b. The left panel on the software should say READY at the top and have two boxes: one that says Sample Information and one that says SOM.
- c. Click CHANGE in the SOM box on the left (or go to SETTINGS→Change SOM).
- d. In the 'control mode' box, make sure TIME is clicked and set to 3 seconds (our default). The number of runs should be set to 1. The two check boxes should be unchecked.
- e. In the second 'box' click the THRESHOLD button to check the background noise of the diluent. Click MEASURE NOISE LEVEL. The level should be somewhere

less than 14  $\mu\text{m}$  if the aperture is okay and the diluent is okay. If not, ask for help. Click OK. I usually set the Sizing and Counting Threshold at 14  $\mu\text{m}$  (this is where it starts recording data), only because it is consistent with my other runs. You should set this wherever you want but I suggest you set it below the cutoff you will use in data analysis.

- f. In the box that starts 'After Each Run' the only box that should be checked is 'save file' and 'include pulse data'. Click the DIRECTORY button to tell the machine where to save your files. Browse for the folder you made in 'b' and select it.
- g. Go back to the very top of the Standard Operating Method window and click OK.
- h. In the 'Sample Information' box on the top left panel, click CHANGE. This should pop up a new window. Enter in the sample name in the Group ID box (i.e. SUM149, control etc.) and enter in the replicate number in the Sample ID box (i.e. 1, A etc.). Click OK. The current file naming system is set up to name files as GroupID\_SampleID\_Date\_run number.#M3. When collecting 6 runs for each replicate the samples will be named SUM149\_A\_10 Dec 2008\_01.#M3, SUM149\_A\_10 Dec 2008\_02.#M3 etc.

### 3. Diluent Quality Control II

- a. It is imperative that the diluent be reading "quietly" when the diluent is run by itself. Change the sample name to something like 'background' so this data is distinguished from your sphere data.
- b. Make sure there is at least 20 ml of diluent in the coulter bucket. From the top menu panel click the green START arrow. You should see a graph pop up with red bars indicating counts. There is also a small window that plots counts in black lines on the left panel. You should get somewhere in the range of 0-15 counts if the diluent is behaving properly. Note that sometimes it takes several runs for the diluent to settle down. I think there is sometimes bubbles in the aperture that make it have higher counts.
- c. Click start again to do a second run. Refill the bucket with 12.5 ml of diluent by lowering the stage, pipeting diluent into the bucket along the wall of the bucket, and raising the stage. Click start again to do another run. Repeat this process to see if the counts settle in to the range of 0-15, refilling the bucket after every two runs.
- d. If the counts remain high there are a few options to try.
  - i. Make sure that you are not introducing bubbles when pipetting, watch as you aspirate into the pipet and pipet out into the bucket for excessive bubbling...done slowly and carefully this should not be a problem.
  - ii. Try changing to a new bucket of diluent, run again.
  - iii. When the bucket is full, try flushing the aperture tube (SYSTEM→FLUSH APERTURE TUBE), this will aspirate into the aperture and then flush into the bucket. Change to a new bucket of diluent and run again at least twice.

- iv. If none of the above works, try a new bottle of diluent. Old bottles sometimes will start to have higher counts if there is contamination.
- e. Note that if you ever do run out of diluent during a run and introduce bubbles you must refill the bucket and then flush the aperture tube or it won't run. Change to a fresh bucket after every aperture flush (it just spits bubbles into the diluent).
- f. Leave the aperture immersed in the diluent and go prepare samples.

**A NOTE on the waste tanks.** There is an internal waste tank that is monitored by the blue bar at the bottom left of the screen. This empties to the external waste tank when you go to SYSTEM→EMPTY WASTE TANK. Make sure you monitor the levels in the waste tank and empty it when it gets near full (a red bar appearing means you are full). If you do not do this and the tank becomes full during a run it will stop everything and give you a warning message. It will often not record the data from that run even though it has sucked up the sample. This usually makes me pretty cranky when this happens so prevention is the best cure! I keep an eye on it and check it before I start a sample. It helps to empty it before you run any samples after you have filled the system as this process seems to fill up the waste tank. I usually empty the external waste tank into the sink when it gets near full or when I am done with the machine, run plenty of water to flush.

#### 4. Sample Preparation

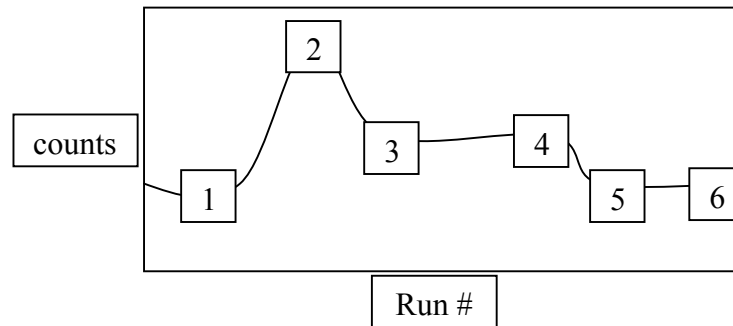
The method of preparation will depend on your preference, as long as you are consistent with how you run your data, it shouldn't really matter. The following instructions are the way that I run my data. I typically will prepare only 6-12 samples at time to avoid having some samples sit for extended periods of time.

- a. Collect spheres to a 15 ml conical tube, wash out the well with PBS and add this to the conical tube.
- b. Spin down spheres at 6-800 rpm for 4-5 min. Aspirate off the media and resuspend spheres in about 1 ml of media. Note I have found that my spheres will disintegrate after extended incubation in PBS so I recommend you resuspend in media.
- c. Bring samples and a tube of media for a control over to the multisizer, where you should have prepared enough buckets with 20 ml of diluent for your samples plus a few extra.

#### 5. Running your samples

- a. Change the sample name to Media control (or something like that). To change the sample name after a run, click the RESET button on the left panel near the bottom. This will bring you back to the panel where you see the Sample Information box, click CHANGE, and type in your new sample name.
- b. With a 1 ml pipet or a p1000, pipet 1 ml of media into the 20 ml of diluent in a bucket, inserting the pipet tip into the diluent so the sample mixes well.

- c. Place the bucket on the stage. Do 2 runs, add 12.5 ml diluent, do 2 more runs, add 12.5 ml diluent. This is 4 runs total; for the media control, you should see a low number of counts (50-60 max in any one run). I save these runs and use them to subtract out background from my samples during data analysis.
- d. Periodically change to a fresh bucket of diluent and run a blank sample. You can change the sample name for this if you want. I usually just note that the 5<sup>th</sup> run for any sample is my blank. The counts should be in the 0-15 range again. I usually just add more diluent to this bucket and re-use it as my blank between runs but if you notice the counts creeping up, change to a fresh bucket.
- e. Proceed with your samples, repeating steps a-c for each, running a blank is optional between samples. I have found that it is nearly always 0-15 counts and therefore it is a waste of diluent to run a blank between each sample but periodically checking the diluent performance may be warranted if your samples seem to be behaving oddly.
  - i. Note: you should see decay in the counts with your subsequent runs. If you don't, something is wrong (unfortunately, for some cell lines, it behaves this way and I don't know why). I recommend you do all 4 runs for a few samples to see if it is behaving properly before resorting to alternate methods. I typically see the counts behave with the following pattern (give or take; note schematic is from when I used to do 6 runs per sample, I have found runs 5 and 6 have a lot of noise in them so have stopped at 4):



- ii. The media will sometimes have a lot of counts in runs 5&6 or it won't decay, I generally take these as bogus and use the first 4 runs to estimate a background subtraction factor (it is an estimate after all).
- iii. Note that you end up reading about 90% of your input by doing four to six runs. More runs will give you more counts but it starts getting to a point where it is just noise and is not worth the time. Thus, it will still be an estimate of the total number of spheres counted (though a hand count will also be an estimate, in some respects).
- iv. If you start seeing a lot of counts in the blanks, try some of the tips in part 3d.

## 6. Exchanging the diluent for Coulter Cleanz; shutting down the machine

Repeat the procedure in step 1, except you are now exchanging the diluent for the Coulter Cleanz. There should be a small bottle of Coulter Cleanz that you can use to fill the bucket between FILL SYSTEM steps. More Coulter Cleanz is stored on the shelf to the right of the Multisizer. Please store upright as these leak!

**7. Data retrieval and analysis (current method, I think there is a way to set the files to save as converted in the first place but I haven't tested it yet)**

- a. Before you begin, make a copy of your data folder and use the copy to manipulate the files (in case you need to go back to the originals)
- b. Overlay the 4 files from each sample: Go to FILE→OVERLAY and select the files to overlay (ex. Sample A 1-4). Click ADD, then OK. A graph should appear with 4 sets of lines corresponding to your 4 runs. Note you can do this with more than 4 samples since this is just converting pulses to size and not adding runs. I will often open up as many as it will let me.
- c. In the graph window go to ANALYZE→Convert pulses to size. In the new window:
  - i. Change Log Diameter to Lin Diameter
  - ii. Check the box to do all runs
  - iii. Make 300 bins between 14  $\mu\text{m}$  (or wherever you started collecting data) and 336  $\mu\text{m}$  (this is roughly 2-60%, the accurate range of the aperture). If you want 300 bins with 1  $\mu\text{m}$  increments use 20-320 as your range.
  - iv. Coincidence correction should be on, Multisizer II edit off

Click OK, the lines in the graph should convert to steps.

- d. In the graph window, go to RUN FILE→Save All.
- e. Back in the main Multisizer window go to FILE→Add runs. Select the 4 runs from one of your samples that you just converted. Click ADD, then OK. You should now see one red step graph that represents your added runs.
- f. In the graph window, got to RUN FILE→Save As. Give this file a new name to distinguish it from the individual files.
- g. From RUN FILE→Export Data. Check the Listing box (leave the others as is). This will now export the data as a CSV file that can be opened in Excel.
- h. In Excel, open the CSV file and you should see data as follows:

Bin Number	Bin Diameter (Lower) $\mu\text{m}$	Diff. Number	Diff. Volume %
1	14	88	1.12643
2	15.0733	88	1.39408
3	16.1467	80	1.54648
4	17.22	79	1.84059



The bin number refers to the 300 bins you made. Bin diameter (lower) is the lower size limit of the bin. So in Line 1, 88 (Diff. Number) refers to the number of counts between 14  $\mu\text{m}$  (the lower limit of the bin) and 15.0733  $\mu\text{m}$  (the lower limit of the next bin). I disregard Diff. Volume %.

To get an estimate of the total counts, sum the numbers in the Diff. Number column greater than your size cutoff of choice (ex. 40  $\mu\text{m}$ ). I usually will do the same for the media control and subtract this out as background.